Pak. J. Bot., 53(6): 2099-2109, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-6(40)

SELECTION AND EVALUATION OF REFERENCE GENES BY RT-qPCR ANALYSIS IN *ACONITUM VILMORINIANUM* KOM.

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Abstract

Aconitum vilmorinianum Kom. is one of the most important traditional Chinese medicine. However, there are few reports on the molecular biology of *A. vilmorinianum*. Fluorescence quantitative real-time polymerase chain reaction (RT-qPCR) is widely used detection method for target gene expression analysis, but the selection of the best reference gene is very important for the accuracy of the method. In this study, based on transcriptome data six candidate internal reference genes were selected for the first time and their expression stability in root, stem, leaf and flower of *A. vilmorinianum* was systematically evaluated by geNorm, Normfinder and BestKeeper. The results showed that *MDH*, *GAPDH* and *18S RNA* could be used as reference genes in root; *MDH* and*18S RNA* can be used as reference genes in stem; *MDH*, *GAPDH* and *β-TUB* could be used as reference genes in leaf, and *GAPDH*, *18S RNA* and *ACT* could be used as reference genes in flower. In addition, the applicability of the selected reference genes was verified by detecting two functional genes related to alkaloid metabolism, namely hydroxymethylglutaryl-CoA reductase (*HMGCR*) and phosphomevalonate kinase (*PMK*). The expression profiles of the *HMGCR* and *PMK* genes were similar after standardization to the stably expressed reference genes *MDH*, *GADPH*, *18S RNA* and *ACT*. However, when *UBQ* was used as the reference gene, the expression profiles of *HMGCR* and *PMK* genes were different. These results provide useful information for obtaining reliable RT-qPCR standardized data in genetic research on *A. vilmorinianum*.

Key words: Gene expression, *Aconitum vilmorinianum* Kom., Reference gene, RT-qPCR Abbreviations.

Introduction

Aconitum vilmorinianum belongs to the family Ranunculaceae. There are many species in the genus *Aconitum*, and about 200 species in China (Li & Kadota, 2001). The Hengduan Mountains and Jinsha Jiang River Valley regions in Yunnan Province are the center of abundance, differentiation and preservation of primitive groups of *Aconitum* in modern distribution, where there are 66 species, 25 varieties and 4 forms of *Aconitum. A. vilmorinianum* is concentrated as mountain shrubs found at 2,100-3,000 m above sea level in central and western Yunnan, as well as Western Guizhou. As *A. vilmorinianum* contains diterpenoid alkaloids, such as Yunnan aconitine, Huangcao aconitine A, and bulleyaconitine A, it has antirheumatic activity, improves human meridians, relieves swelling and pain, dispels cold, activates blood circulation, and inhibits tumor growth (Li *et al*., 2016). As an important traditional Chinese medicine, the root of *A. vilmorinianum* is the main raw material of Yunnan Baiyao, Bulleyaconitine A Tablets and other well-known Chinese medicines. The reports on *A. vilmorinianum* research are mainly focused on chemical constituents and pharmacological effects. Molecular biology research started relatively late. So far, there is no report on the selection of reference genes of *A. vilmorinianum*. With the development of the molecular biology of *A. vilmorinianum*, the screening of selecting stable reference genes suitable for this species is of great significance for the genes expression analysis and functional characterization of target genes.

Fluorescence quantitative real-time polymerase chain reaction (RT-qPCR) is a method to monitor the whole reaction process by adding fluorescent dye or fluorescent probe into the PCR reaction system for the accumulation of fluorescent signals. The target genes were quantitatively analyzed by a standard curve. The technique has high sensitivity, specificity and accuracy. Because of its high detection efficiency and wide range, It has been widely used in plant gene expression analysis. (Huggett *et al*., 2005; Dussault & Poullot, 2006). However, since the actual expression of genes is affected by the quality of the extracted RNA, the efficiency of transcription and other factors, so it is necessary to introduce internal reference genes to correct and standardize the expression results (Bustin, 2002; VanGuilder *et al*., 2008). The ideal reference gene should be stably expressed in all tissues and all developmental stages. However, plethora of studies have shown that the expression of many classical reference genes varies in different species, tissues and conditions (Bustin *et al*., 2005; Nolan *et al*., 2006; Guénin *et al*., 2009; Artico *et al*., 2010; Ali *et al*., 2018). As a reference gene, β-*ACT* (β-actin) can be used for functional gene analysis of *Panax ginseng* (Hou *et al*., 2014). *EF-1β* (elongation factor-1β) and *ACT-2* (actin-2) are more stable in *Anoectochilus roxburghii* (Lin *et al*., 2018). In RT-qPCR analysis of *Dendrobium officinale*, *EF-1α* and18S ribosomal RNA (*18S RNA*) are better reference genes (Zhang *et al*., 2013). *ACT* (actin), *EF* (elongation factor) and *18S RNA* are more stable in root and leaf of *Helianthus tuberosus* (Song *et al*., 2018). The expression

of *TIP41* (tonoplast intrinsic protein 41) and *UBQ10* (ubiquitin 10) was stably expressed in the floral organs of *Rehmannia glutinosa*, while the expression of *TIP41* and *UBQ5* (ubiquitin 5) was stably expressed in the root, stem and leaf (Hou *et al*., 2011). *ACT* (actin) showed good stability in the fruit development stage of *Lycium barbarum* (Zeng *et al*., 2014). Thus, different species and tissues have, different reference genes. Therefore, it is very important to select the suitable reference genes according to the different experimental materials when using RT-qPCR for the anlaysis of the gene expression in different species and tissues.

Recently, we uses high-throughput sequencing technology to determine the transcriptome profiles of root formation of genes in *A. vilmorinianum*. The sequences are uploaded to NCBI (Submission number: PRJNA667080). The transcriptome data provide abundant information for selecting reliable reference genes. By integrating the reported RT-qPCR reference genes, *GAPDH* (glyceraldehyde phosphate dehydrogenase), *ACT*, *MDH* (malate dehydrogenase), *UBQ*, *β-TUB* (β-tubulin) and *18S RNA* （18S ribosomal RNA）were selected as appropriate reference genes. The expression stability of the six candidate reference genes in root, stem, leaf and flower of *A. vilmorinianum* was analyzed by RT-qPCR, using BestKeeper, GeNorm and NormFinder software (Vandesompele *et al*., 2002; Andersen *et al*., 2004; Pfaffl *et al*., 2004). The expression of two functional genes, namely *HMGCR* (hydroxymethylglutaryl-CoA reductase) and *PMK* (phosphomevalonate kinase), which are key genes for alkaloid metabolic pathways in *A. vilmorinianum*, was used to verify the reliability of the reference genes for RT-qPCR analysis of root, stem, leaf and flower-related genes. The results lay a foundation for research of gene expression analysis in the alkaloid metabolic pathways and molecular biology characterization of their components in plant, and also provide a reference for the selection of reference genes in *Aconitum* species.

Material and Methods

Plant materials: Plants of *Aconitum vilmorinianum* cultivated in the town of Malutang, Luquan County, Yunnan Province, China were transplanted in the greenhouse at the University of Science and Technology in Kunming, Yunnan, China. The plants with good growth condition at the flowering stage were selected. Roots, stems, leaves, flowers were separately collected, along with roots in the early, middle and formative stages of development. All samples were washed with pure water, cut into small pieces after water was absorbed, wrapped in tin foil paper, labeled, and put in liquid nitrogen immediately, and stored for subsequent RNA extraction at -80℃.

RNA isolation and reverse transcription: RNA was extracted using the Trizol method. The experimental materials were ground in liquid nitrogen, mixed with 1 ml Trizol reagent and 200 μl trichloromethane in 2.0 ml centrifuge tube. After vigorous shaking and mixing, the sample was placed on ice for 5 minutes, and centrifuged at 4℃ and 12 000 rpm for 15 minutes. About 450 μl supernatant was taken to new centrifugal tube and 400 μl trichloromethane was absorbed to the supernatant. Shake the sample vigorously, place it on ice for 5 minutes, and centrifuge at 4℃ and 12000rpm for 15 minutes. Transfer about 400 ml of supernatant to a new centrifuge tube, add 400 μ l isopropanol, shake well and mix, then place it in the refrigerator for 30 minutes, then centrifuge at 4℃ and 12000 rpm for 30 minutes. Take out the liquid in the tube, add 500μl 75% ethanol, and centrifuge at 4℃ and 7500 rpm for 15 minutes. Repeat this step and wash three times. Pour out the liquid in the tube, dry the centrifuge tube and add 20 μL DEPC (diethyl pyrocarbonate) water into the tube. Total RNA was extracted, and the purity and integrity of RNA were detected by agarose gel electrophoresis of 1.2%. According to the operation method of the reverse transcription kit（TransScript II OneStep gDNA Removal and cDNA Synthesis SuperMix, TaKaRa, Dalian, China, the reverse transcription system was 20 μL. The first strand of RNA was synthesized by reverse transcription, and the cDNA products were directly used or stored in refrigerator at -80℃.

Primer design of candidate reference genes and PCR conditions: According to the transcriptome sequencing data of root development of *Aconitum vilmorinianum* (unpublished), *GAPDH, MDH, β-TUB, ACT, 18S RNA* and *UBQ* genes were selected as candidate reference genes. The primers were designed by Primer Premier 5.0 (Udvardi *et al*., 2008) software (Table 1) and were synthesized by Suoqin Biotechnology Co., Ltd.

RT-qPCR were performed using CFX96TM Real-Time System (Bio-Rad Laboratories, CA, USA). Each 20 μl PCR reaction mixture constitutes SYBR Premix Taq (10 μl), cDNA (1 μl), forward and reverse primer (0.8 μl respectively) and ddH_2O (7.4 μl). Cycling conditions involved 10 s at 94˚C followed by 40 cycles of 94˚C for 15 s, 53˚C for 34 s and 72˚C for 30 s. Each reaction was performed in triplicate.

Stability analysis of reference gene: The cDNAs of roots, stems, leaves and flowers were diluted by 5 gradients, 5 times each gradient, i.e. the concentration of the cDNAs was 1, 1/5, $1/5^2$, $1/5^3$ and $1/5^4$ times of the initial concentration, respectively. Each candidate reference gene was amplified by RT-qPCR according to the concentration gradient of cDNA dilution (Bustin, 2002). Using template concentration as abscissa and Ct value as ordinate, the standard curve was drawn, and the slope K and correlation coefficient R^2 were obtained. The primer amplification efficiency (E) was calculated by formula $E = 10^{-1/k} - 1 \times 100\%$ (Sinha *et al*., 2015). The stability of reference gene expression was analyzed according to the results of three software programs: Bestkeeper, Genorm and Normfinder. For the evaluation of expression stability, the BestKeeper program uses an index to be calculated according to standard deviation (SD) and percentage covariance (CV) values. The GeNorm program was characterized with lower expression stability value (M) for Stable expression genes based on the pairwise (Silver *et al*., 2006). The NormFinder program can assess degrees of variance within and between groups, and the gene with the lowest value of stability expression is ranked as the best.

Table 1. Primer sequences for real-time qPCR.

Primer	Sequence (5′-3′)	Annealing temperature (℃)	Product length (bp)
GAPDH	F-GCTATCAAGGAGGAATCT	50.3	139
	R-AATATGCTGGACCTACTG	50.3	
ACT	F-CTGTATGTCGTGGTCTTC	52.6	148
	R-CAATGGAACTGGAATGGT	50.3	
MDH	F-CCAGCCTGTAATTCTTCA	50.3	187
	R-CTATCAACTCCATCTTCACT	51.3	
UBQ	F-TGTTGCTGAAGATATGTT	48.1	125
	R-CTACCATTGCTGTTGATA	45.8	
β-TUB	F-TTCTTCTCATTAGTTCTTG	45.8	173
	R-ACACCTATATTGACGAAT	46.5	
18S rRNA	F-AGTTGGCTTCTTCGTTGT	54.2	186
	R-CATCTTGGTAAACCTTGGTATAG	50.3	

Validation of candidate reference gene: In order to verify the stability of reference genes, according to the analysis results of Bestkeeper, GeNorm and Norm-finder, expression level of two functional genes related to alkaloid metabolism, *HMGCR* and *PMK*, in roots, stems, leafs and flowers, were detected by $2^{-\Delta\Delta ct}$ method (Manuka *et al*., 2018). In addition, correlation coefficients between the relative expression patterns of *HMGCR* and *PMK* in root development and *FPKM* of transcriptome in root were analyzed to further verify the stability of the selected reference genes in root. The primers of *HMGCR* were 5'-ATGGTGATGGTGATGGTG (forward) and 5'-ATTCCTCCTCCTGTCTCT (reverse), and the primers of PMK were 5'-CCGATTGAGCCAGAACTAC (forward) and 5'-AACTCCTGCCACAAGAAC (reverse).

Results

RNA quality and primer specificity detection: After extraction of total RNA from root, stem, leaf and flower of *A. vilmorinianum*, the integrity and purity of RNA were assessed by 1.2% agarose gel electrophoresis analysis. The results clearly showed the expected RNA electrophoresis bands for intact total RNA and no visible contaminant was observed (Fig. 1). This shows that the purity is high, and these RNA samples can be used in subsequent experiments. The PCR products obtained with all the six primers used in the experiment produced only one band when checked by electrophoresis, which indicated that each of these primers could specifically amplify the corresponding reference gene, and there was no primer dimer formation (Fig. 2). The qRT- PCR melting curves showed that the six candidate reference genes had a single main peak, and repeatability between the same samples was good (Fig. 3).

Primer amplification efficiency analysis: The amplification efficiency (E), correlation coefficient (R^2) and standard curve slope of *GAPDH*, *ACT*, *MDH*, *β-TUB*, *18S RNA* and *UBQ* were determined by RT-qPCR using a 5-fold concentration gradient dilution of cDNA from root, stem, leaf and flower. In root, *MDH* had the highest amplification efficiency at E=97.911% (R^2=1); *ACT* and *β-TUB* had the lower amplification efficiency at E=79.493% (R^2=1) and E=88.414% (R^2=0.999), respectively. In stem, *18S RNA* had the highest amplification efficiency at E=90.884% (R^2= 0.988); *β-TUB* and *UBQ* had the lower amplification efficiency at E=75.296% (R^2= 0.9999) and E=78.595% (R^2= 0.9995), respectively. In leaf, the amplification efficiency of *β-TUB* was the highest at E=80.376% (R^2=1); the amplification efficiency of *MDH* and *18S RNA* was lower at E=53.982% (R^2= 0.9972) and E=66.74% (R^2= 0.9936), respectively. In flower, the amplification efficiency of *18S RNA* was the highest at E=94.281% (R^2= 0.9969); the amplification efficiency of *β-TUB* and *GAPDH* was lower at E=73.361% (R^2= 0.9983) and E=78.836% (R^2= 0.9998) respectively (Table 2). All of these reference genes can meet the requirement of RT-qPCR analysis for primer amplification efficiency.

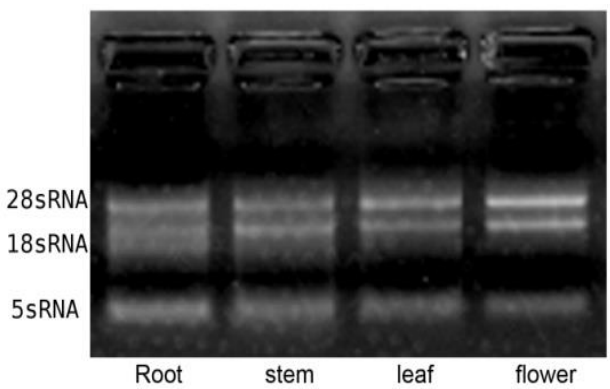

Fig. 1. Agarose gel electrophoresis analysis of total RNA extracted from different tissues of A. vilmorinianum.

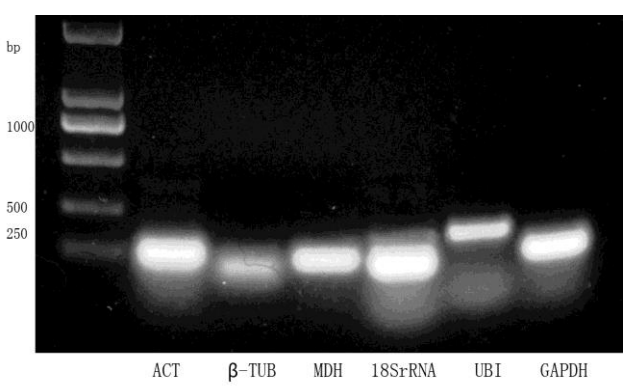

Fig. 2. Amplified products of the six candidate reference genes detected by agarose gel electrophoresis.

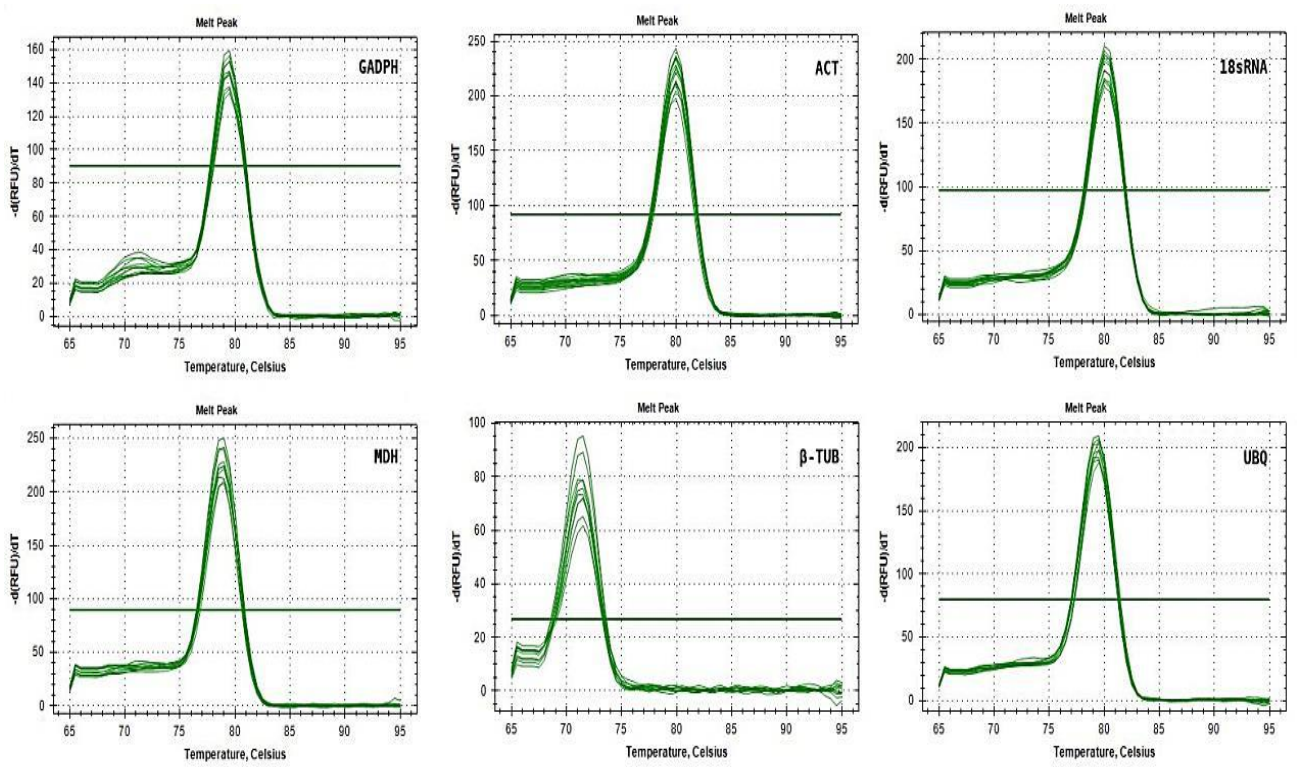

Fig. 3. Melting curves of the six candidate reference genes.

Table 2. Description of candidate reference genes and RT-qPCR efficiency.

Tissue	Index of Amplification	GAPDH	ACT	MDH	β-TUB	18S rRNA	UBQ
Root	Amplification efficiency (%)	95.824	79.493	97.911	88.414	91.441	94.869
	Correlation coefficient (R^2)	0.9998	1	1	0.999	0.8989	1
	Slope (k)	-1.0727	-1.402	-0.5827	-1.057	-0.9837	-1.3087
Stem	Amplification efficiency (%)	80.754	83.754	83.715	75.296	90.884	78.595
	Correlation coefficient (R^2)	0.8973	0.9965	0.9996	0.9999	0.988	0.9995
	Slope (k)	-1.3973	-1.267	-1.2677	-1.6773	-0.928	-1.231
Leaf	Amplification efficiency (%)	72.246	78.493	53.982	80.376	66.74	79.948
	Correlation coefficient (R^2)	0.9999	0.992	0.9972	1	0.9936	0.9995
	Slope (k)	-1.7483	-1.159	-2.2683	-1.4073	-1.5167	-1.2393
Flower	Amplification efficiency (%)	78.836	83.817	88.086	73.361	94.281	88.323
	Correlation coefficient (R^2)	0.9998	1	0.9979	0.9983	0.9969	0.9979
	Slope (k)	-1.4407	-1.2333	-1.082	-1.5477	-0.8247	-1.208

Analysis of expression level of candidate reference genes: The higher the threshold cycle (Ct) value, the lower the expression of target genes. The Ct values of the six candidate reference genes in various tissues, namely root, stem, leaf and flower (Fig. 4), showed their expression levels. The Ct values of the six candidate reference genes expressed in root, stem, leaf and flower tissues were between 19 and 35. The *ACT* expression level was the highest in root, and that of *GADPH* was the lowest. In stem, the expression level of *ACT* was the highest and that of *UBQ* was the lowest. In leaf and flower, the expression level of *18S RNA* was the highest and that of *UBQ* was the lowest. There is no constant expression level of the six candidate reference genes in root, stem, leaf and flower. Therefore, it is necessary to select suitable reference genes using statistical methods.

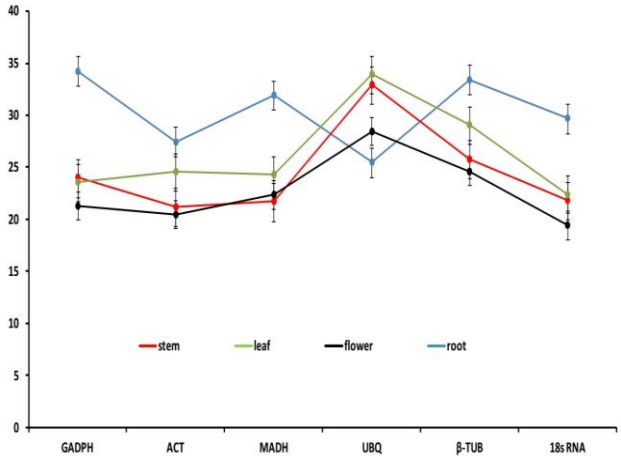

Fig. 4. Ct value of the six candidate reference genes in root, stem, leaf and flower.

Stability of candidate internal reference gene expression

Best keeper software analysis: The BestKeeper software is directly used to analyze the Ct value of gene expression. The stability of gene expression is judged by the standard deviation (SD) and coefficient of variation (CV) of the Ct value of the reference gene. The smaller the SD value, the more stable the expression is (Zhou *et al*., 2018). The default threshold of the program is 1. When the SD value is greater than 1, then the gene expression is considered to be unstable, the lower the SD value and CV value, the more stable the gene expression is. The results showed that the SD values of the six candidate reference genes were all less than 1, and all the candidate reference genes met the criteria for reference genes (Table 3). *MDH* (CV+SD=0.08%+0.03) had the most stable expression in root, followed by *GADPH*. The order of stability of the six candidate reference genes was *MDH> GADPH>18SRNA> ACT>UBQ>β-TUB*. *MDH* (CV+SD=0.52%+0.11) showed the most stable expression in stem, followed by *β-TUB*. The order of stability of the six candidate reference genes was *MDH>β-TUB>UBQ>18S RNA> GADPH>ACT*. The expression of *MDH* (CV+SD = 0.21%+0.05) was more stably expressed than the other reference genes, followed by *GADPH*. The order of stability of the six candidate reference genes was *MDH>GADPH>18S RNA>β-TUB>ACT>UBQ*. In flower, expression of *GADPH* (CV+SD=0.52%+0.12) was the most stably expressed gene, followed by *β-TUB*. The order of stability of the six candidate reference genes was *GADPH>β-TUB>UBQ>MDH>ACT>18S RNA*.

GeNorm software analysis: GeNorm is based on the average expression stability, M, to determine the stability of candidate reference genes. The Ct values obtained by RT-qPCR experiments need to be transformed by the ΔCt method before they can be used for analysis. It is generally accepted that the M value equal to 1.5 is the limit of stable gene expression. Genes with an M value greater than 1.5 are not suitable as reference genes. The higher the M value, the lower the stability of the reference gene expression; the smaller the M value, the more stable the reference gene expression (Vandesompele *et al*., 2002). The results showed that the M values of the six candidate reference genes were all less than 1.5, indicating that they all met the requirements for use as reference gene. In root, *MDH* (M=0.317) and *GADPH* (M=0.317) were the most stably expressed genes, while *β-TUB* (M=0.595) was the most unstably expressed gene. In stem, the most stably expressed genes were *18S RNA* (M=0.238) and *MDH* (M=0.244), and *ACT* (M=0.504) was the most unstably expressed gene. In leaf, *MDH* (M=0.142) and *18S RNA* (M=0.147) were the most stably expressed genes, and *UBQ* (M=0.256) was the most unstably expressed genes. In flower, *GADPH* (M=0.326) and *MDH* (M=0.360) were the most stably expressed genes, while 1*8S RNA* (M=0.586) was the most unstably expressed gene (Fig. 5). GeNorm can also analyze the optimal number of reference genes by calculating the paired variation value $V_{n/n+1}$ of candidate reference genes. By default, the critical value of $V_{n/n+1}$ is 0.15. When $V_{n/n+1}$ is less than 0.15, n reference genes can meet the requirements of reference genes without introducing the n+1 gene. The results showed that the values of $V_{n/n+1}$ < 0.15, $V_{2/3\ (root)}$, $V_{2/3\ (stem)}$, $V_{2/3\ (leaf)}$ and $V_{2/3\ (flower)}$ were 0.062, 0.031, 0.041 and 0.100, respectively, all of which were less than 0.15 (Fig. 5). This indicates that the six reference genes in root, stem, leaf and flower samples meet the requirements of standardization. Selecting a combination of two of the most stable reference genes can accurately correct the expression of the target gene without introducing a third gene for correction. *MDH* and *GADPH* were combined in root, *18S RNA* and *MDH* in stem, *ACT* and*18S RNA* in leaf, and *GADPH* and *MDH* in flower.

NormFinder software analysis: NormFinder calculates the stability of gene expression based on intra-group and inter-group variations. The Ct value of gene expression also needs to be transformed by the Ct method before it can be used for analysis. The larger the stability value (SV), the worse the stability of gene expression and *vice versa* (Wei *et al*., 2013). The results showed that *MDH* (SV=0.03) and *GADPH* (SV=0.03) were the most stably expressed genes in root, while *β-TUB* (SV=0.38) was the most unstably expressed. *MDH* (SV=0.02) and *18S RNA* (SV = 0.04) were the most stably expressed genes in stem, and *ACT* (SV=0.32) was the most unstably expressed. *MDH* (SV=0.01) and *18S RNA* (SV=0.02) were the most stably expressed genes in leaf, and *UBQ* (SV=0.17) was the most unstably expressed gene. *GADPH* (SV=0.06) and *MDH* (SV=0.08) were the most stably expressed genes in flower, while *18S RNA* (SV=0.36) was the most unstably expressed (Table 4).

Validation of the stability of internal reference genes: The results of the analysis by BestKeeper, GeNorm and NormFinder reveal that *MDH*, *GADPH*, *18S RNA* and *ACT* are relatively stable (Table 5). In order to validate the stability of the reference genes, using the ΔΔCT method, the expression levels of two functional genes, namely *HMGCR* and *PMK*, related to alkaloid metabolism were detected using different reference genes (*MDH*, *GADPH*, *18S RNA*, *ACT* and *UBQ*). *MDH* was used as reference gene to detect the expression levels of *HMGCR* and *PMK* during the root development process, and correlation analysis was conducted between the relative expression levels of *HMGCR* and *PMK* and the expression levels of the two genes (FPKM) of the root transcriptome. The results showed that the expression levels of *HMGCR* and *PMK* genes in root, stem, leaf and flower were similar under the standardized treatment with *MDH*, *GADPH*, *18S RNA* and *ACT* (Fig. 6A, B). However, when *UBQ* was used as reference gene, the expression levels of the *HMGCR* and *PMK* genes were significantly different (Fig. 6A, B).

The correlation coefficient between the expression patterns of *HMGCR* and *PMK* genes using *MDH* as the reference gene and the expression pattern of the two genes *FPKM* (Reads Per Kilobase of transcript per Million mapped reads) of the root transcriptome were 1 and 0.997, respectively, which indicates that the expression trends of the *HMGCR* and *PMK* genes in the two modes were consistent (Fig. 6C).

Table 3. The stability of candidate reference genes based on best keeper analysis.

Tissue	Stability rank	1	2	3	4	5	6
Root	gene name	MDH	GADPH	18s RNA	ACT	UBQ	β-TUB
	geo Mean	31.93	34.25	29.69	27.45	25.46	33.42
	ar Mean	31.93	34.25	29.69	27.45	25.47	33.42
	min	31.89	34.19	29.45	27.14	25.19	33.11
	max	31.96	34.31	29.83	27.83	25.98	33.97
	std dev	0.03	0.04	0.16	0.26	0.34	0.37
	CV (%)	0.08	0.12	0.53	0.93	1.02	1.10
Stem	gene name	MDH	β-TUB	UBQ	18s RNA	GADPH	ACT
	geo Mean	21.65	25.75	32.89	21.79	23.95	21.18
	ar Mean	21.65	25.75	32.89	21.79	23.95	21.19
	min	21.48	25.54	32.68	21.59	23.80	20.81
	max	21.75	25.96	33.18	21.94	24.23	21.59
	std dev	0.11	0.14	0.19	0.14	0.19	0.27
	CV (%)	0.52	0.55	0.58	0.62	0.79	1.27
Leaf	gene name	MDH	GADPH	18s RNA	β-TUB	ACT	UBQ
	geo Mean	24.28	23.48	22.39	29.02	24.51	33.91
	ar Mean	24.28	23.48	22.39	29.02	24.51	33.91
	min	24.21	23.41	22.31	28.88	24.31	33.59
	max	24.36	23.57	22.48	29.21	24.62	34.15
	std dev	0.05	0.06	0.06	0.13	0.13	0.21
	CV (%)	0.21	0.25	0.28	0.44	0.54	0.62
Flower	gene name	GADPH	β-TUB	UBQ	MDH	18s RNA	ACT
	geo Mean	21.31	24.61	28.48	22.38	19.42	20.47
	ar Mean	21.31	24.61	28.48	22.38	19.42	20.47
	min	21.13	24.37	28.04	22.05	19.08	20.18
	max	21.45	24.94	28.95	22.61	19.66	21.03
	std dev	0.12	0.22	0.31	0.22	0.23	0.37
	CV (%)	0.55	0.90	0.96	0.98	1.17	1.81

Table 4. The stability expression of the six candidate reference genes in different tissues calculated by NormFinder.

Tissue	Stability rank	1	2	3	4	5	6
Root	gene name	MDH	GADPH	18s RNA	ACT	UBQ	β-TUB
	Stability value	0.03	0.03	0.18	0.25	0.32	0.38
Stem	gene name	MDH	18s RNA	β-TUB	GADPH	UBQ	ACT
	Stability value	0.02	0.04	0.09	0.16	0.26	0.32
Leaf	gene name	MDH	18s RNA	ACT	β-TUB	GADPH	UBQ
	Stability value	0.01	0.02	0.04	0.13	0.13	0.17
Flower	gene name	GADPH	MDH	β-TUB	UBQ	ACT	18s RNA
	Stability value	0.06	0.08	0.18	0.29	0.31	0.36

Table 5. Comprehensive ranking of the expression stability of candidate internal reference genes in different tissues.

Tissue	Stability rank	1	2	3	4	5	6
Root	Gene name	MDH	GADPH	18s RNA	ACT	UBQ	β-TUB
Stem	Gene name	MDH	18s RNA	β-TUB	GADPH	UBQ	ACT
Leaf	Gene name	MDH	18s RNA	ACT	β-TUB	GADPH	UBQ
Flower	Gene name	GADPH	β-TUB	MDH	UBQ	ACT	18s RNA

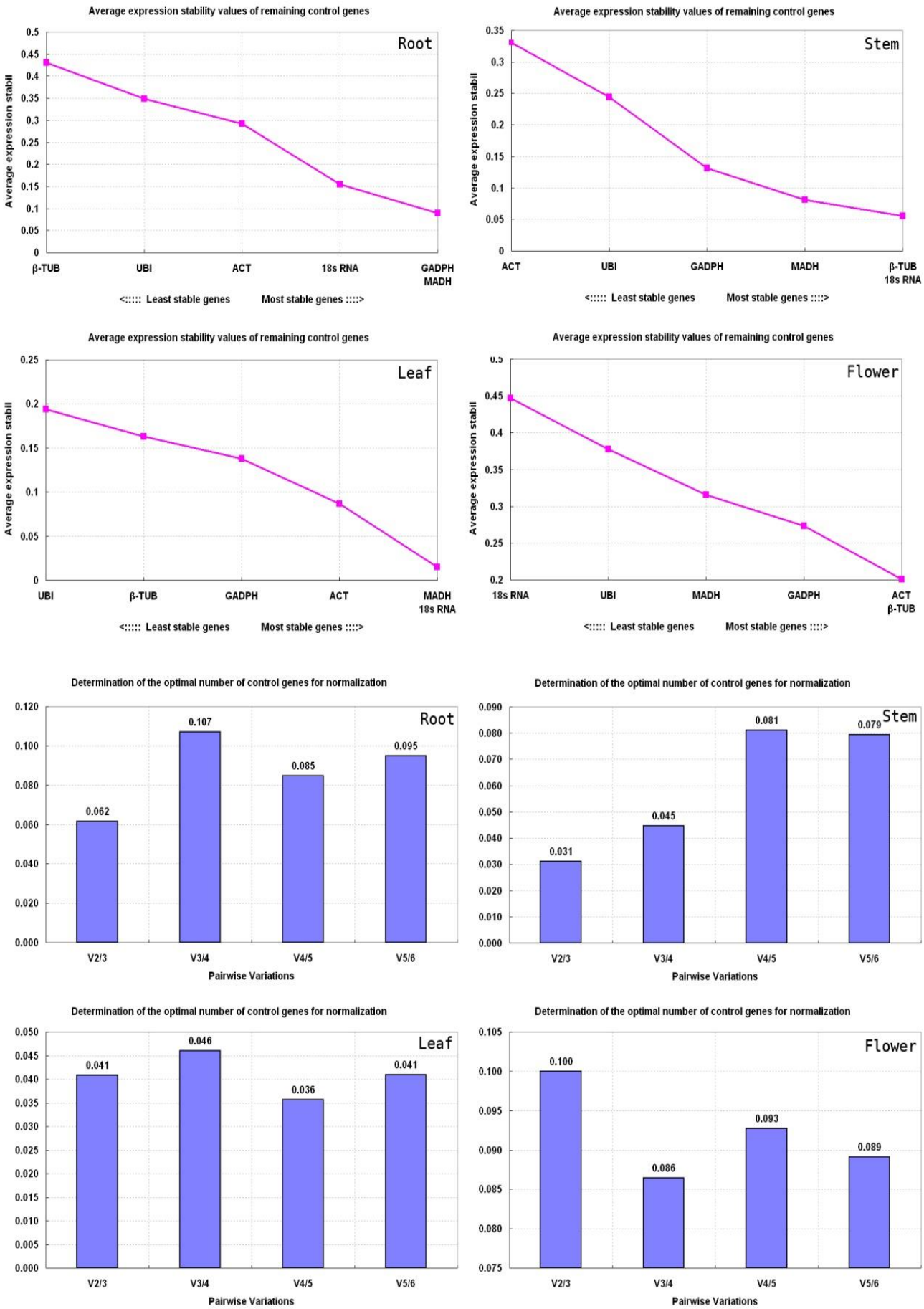

Fig 5. Average expression stability and pairwise variation analysis of the six candidate reference gene according to GeNorm.

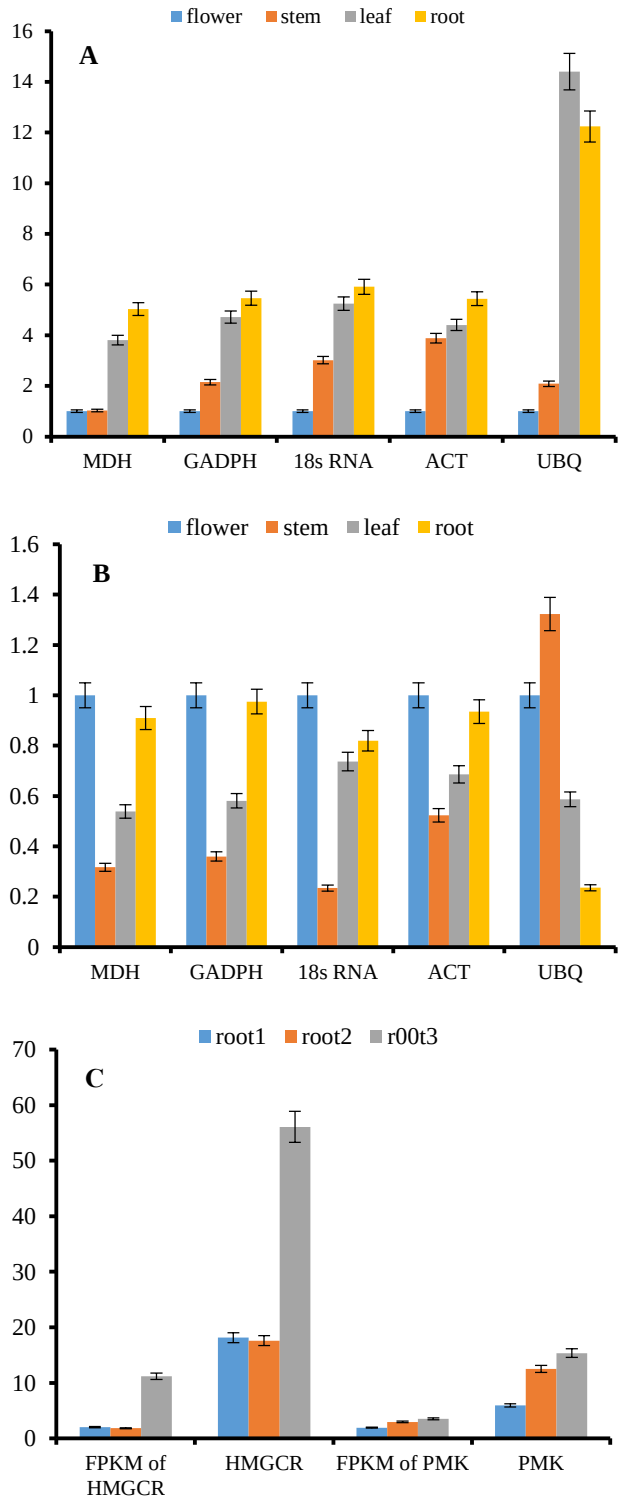

Fig. 6. Relative expression of *HMGCR* and *PMK*.

A: Using *MDH*, *GADPH*, *18S RNA*, *ACT* and *UBQ* as reference genes, the relative expression of *HMGCR* gene in root, stem, leaf and flower tissues was calculated by real-time quantitative PCR. The relative expression levels are presented as the mean+ SD (standard deviation) calculated from three technical replicates. B: Using *MDH*, *GADPH*, *18S RNA*, *ACT* and *UBQ* as reference genes, the relative expression of the *PMK* gene in root, stem, leaf and flower tissues was calculated by real-time quantitative PCR. The relative expression levels are presented as the mean+ SD (standard deviation) calculated from three technical replicates. C: The expression levels of the *HMGCR* and *PMK* genes obtained from the transcriptome data of root formation at the early (root1), middle (root2) and formative stages (root3); the expression levels of *HMGCR* and *PMK* genes at the early, middle and formative stages of root formation were calculated by real-time quantitative PCR using *MDH* as reference gene. Error bars show the standard error calculated from three biological replicates.

Discussion

RT-qPCR has become an important method for gene expression analysis due to its high sensitivity, rapidity, specificity and reliability (Gachon *et al*., 2004; Sinha *et al*., 2015). However, the accuracy of the target gene expression analysis mainly depends on the selection of reference genes. Unstable reference genes can lead to inaccurate gene expression measurement of target genes. Therefore, for accurate and reliable results, before using reference genes to standardize the expression of target genes, it is particularly important to select reference genes that are stably expressed in different tissues (Vandesompele *et al*., 2002; Kong *et al*., 2014). Based on the experimental data, the expression stability of the six candidate reference genes in different tissues of *A. vilmorinianum* was evaluated using the ΔCt method, BestKeeper, GeNorm and NormFinder. The average Ct values of the six reference genes varied from 19.42 (*18S RNA*) to 34.25 (*GAPDH*), and the standard deviation of the Ct values ranged from 0.03 (*MDH*) to 0.37 (*β-TUB* and *ACT*) (Table 3). The different rankings of the reference genes according to the stability obtained by each method may be attributed to the different algorithms used in the four programs (Niu *et al*., 2002).

In root of *A. vilmorinianum*, according to the results of ΔCt calculation, BestKeeper, GeNorm and NormFinder analyses, *MDH*, *GAPDH* and *18S RNA* were relatively stable reference genes. Related studies have found that *GAPDH* can be stably expressed in root. For example, *GAPDH* can be stably expressed in root of *Andrographis paniculata* (Li *et al*., 2013). In addition, *GAPDH* can be used as a reference gene in root of *Dipsacus asperides* due to its stable expression (Jin *et al*., 2018). The expression of *GAPDH* is also relatively stable in *Bergenia purascens* root (Yin *et al*., 2017). When selecting reference genes in different tissues of *Hippeastrum vittatum*, *GAPDH* was found to be a suitable reference gene in root. However, in root of *Helianthus tuberosus* and *Anoectochilus roxburghii*, the expression of *GAPDH* was not stable, while the expression of *ACT* was stable (Song *et al*., 2018; Lin *et al*., 2018). Other studies have found that *UBQ* can be stably expressed as a reference gene in root. For instance, *UBQ* is more stable in root of *Rehmannia glutinosa* (Hou *et al*., 2011). Due to the stable expression of *β-ACT* in root of *Panax ginseng* (Hou *et al*., 2014) and *Psammosilene tunicoides* (Li *et al*., 2016), *β-ACT* is regarded as a reference gene. The most stable expression of *18S RNA* was found in root of *Asarum sieboldii* (Zhao *et al*., 2017), *Lonicera macranthoides* (Cai *et al*., 2016) and *Saussurea medusa* (Yin, 2017). In addition, it has also been found that

25S RNA was more stable in young roots of *Helianthus tuberosus* (Song *et al*., 2018). The stability of *TUB* expression was the best in root of *Polygonatum sibiricum* (Wang *et al*., 2017). In root of *Bupleurum chinense*, the expression of *β-TUB* was stable but the expression of *18S RNA* was unstable (Dong, 2008). Also, *ACT* was the most stable gene in root of *Dactylis glomerata* under drought stress and salt stress (Jiang *et al*., 2014), and *EF-1α* was the most stable gene in root of *Dioscorea composita* under drought stress (Zeng *et al*., 2018).

In stem of *Aconitum vilmorinianum*, according to the results of the ΔCt method, *18S RNA* and *ACT* were relatively stable; *MDH* and *β-TUB* were relatively stable according to analysis by BestKeeper; *18S RNA* and *β-TUB* were relatively stable according to analysis by GeNorm; *MDH* and *18S RNA* were relatively stable according to analysis by Norm Finder. The comprehensive results showed that *MDH*, *18S RNA* and *β-TUB* could be used as reference genes in stem. Similar results showed that *18S RNA* could be used as reference gene in stem of *Saussurea medusa* (Yin, 2017), *Asarum sieboldii* (zhao *et al*., 2017), *Dendrobium officinale* (Zhang *et al*., 2013), *Bergenia purascens* (Yin *et al*., 2017) and *Lonicera macthoranthoides* (Cai *et al*., 2016). However, stable expression patterns of *GAPDH* and *UBQ* were found in stem of *Andrographis paniculata* (Li *et al*., 2013). *β-ACT* gene was stably expressed in stem of *Psammosilene tunicoides* and used as reference gene (Li *et al*., 2016). The expression of *EF-1β* and *ACT* was more stable in stem of *Anoectochilus roxburghi* (Lin *et al*., 2018). As the *ACT* expression in the root of *Dioscorea opposite* was relatively stable, *ACT* could be used as reference gene (Gong *et al*., 2016). The *TUB* gene is stably expressed in root and stem tissues of *Polygonatum sibiricum* and *Dioscorea composita* (Wang *et al*., 2017; Zeng *et al*., 2018). In selecting reference genes in *Bupleurum chinense*, it was found that *β-TUB* could be stably expressed in stem and other tissues, and *18S RNA* gene expression was the most unstable (Dong, 2008). The *25S RNA* gene was stably expressed in young stem and root of *Helianthus tuberosus* (Song *et al*., 2018). *EF-1α* and *GAPDH2* can be used as reference genes in stem of *Hippeastrum vittatum* (Liu *et al*., 2018). *UBQ* expression is relatively stable in stem of *Rehmannia glutinosa* (Hou *et al*., 2011). *UBQ2* and *EF-1α* are stable under normal growth conditions of *Atractylodes lancea*, but *EF-1α* expression is the most stable under drought stress (Sang *et al*., 2017).

In leaf of *Aconitum vilmorinianum*, According to the calculation method of ΔCT, β-*TUB* and *UBQ* were relatively stable; according to Bestkeeper analysis, *MDH* and *GAPDH* were relatively stable; according to GeNorm analysis, *GAPDH* and 18S RNA were relatively stable; according to NormFinder analysis, *MDH* and *18S RNA* were relatively stable. The comprehensive results showed that *MDH*, 18S RNA and *ACT* could be used as reference genes.Similarly, the expression pattern of *GAPDH* was stable in leaf of *Andrographis paniculata* (Li *et al*., 2013), stable in young and mature leaf of *Bergenia purpurascens* (Yin *et al*., 2017), and stable in *Polygonatum sibiricum* (Wang *et al*., 2017). In leaf of *Bupleurum chinense*, *TUB* expression was stable while the expression of *18S RNA* gene was unstable (Dong, 2008). However, 18 S RNA was stably expressed in leaf of *Saussurea medusa* (Yin, 2017), *Dendrobium officinale* (Zhang *et al*., 2013), *Asarum sieboldii* (zhao *et al*., 2017) and *Lonicera macranthoides* (Cai *et al*., 2016) as reference gene, while *β-ACT* gene was stably expressed in leaf of *Psammosilene tunides* (Li *et al*., 2016), and *ACT* was stably expressed in *Anoectochilus roxburghi* (Lin *et al*., 2018) and *Dioscorea opposite* (Gong *et al*., 2016). The expression level of *UBQ* in leaf of *Rehmannia glutinosa* was relatively stable (Hou *et al*., 2011). In addition, *25S RNA* was found to be more stable in leaves of *Helianthus tuberosus* (Song *et al*., 2018), and EF-1α was more stable in leaves of *Dioscorea composita* under drought stress (Zeng *et al*., 2018).

In flower of *A. vilmorinianum*, according to the ΔCt method, expression of *18S RNA* and *UBQ* was relatively stable; according to BestKeeper analysis, expression of *GAPDH* and *β-TUB* was relatively stable; according to GeNorm analysis, expression of *ACT* and *β-TUB* was relatively stable; according to NormFinder analysis, expression of *GAPDH* and *MDH* was relatively stable. The comprehensive results showed that *GAPDH*, *β-TUB* and *MDH* can be used as reference genes. Similar studies have found that *GAPDH* is more stably expressed in flower of *Andrographis paniculata* (Li *et al*., 2013). The expression pattern of *GAPDH* is also stable in flower organs of *Hippeastrum vittatum* and can be used as reference gene (Liu *et al*., 2018). It was also found that *18S RNA* was stably expressed in flowers and other tissues by selecting reference genes of *Lonicera macranthoides* (Cai *et al*., 2016) and *Saussurea medusa* (Yin, 2017). *ACT* was found to be stably expressed in flower organs of *Lonicera japonica* and was used as reference gene (Liu *et al*., 2017). Additionally, *GAPDH* and *ACT* were more stably expressed in flower of *Herbaceous peony* (Li *et al*., 2017). The expression of *ACT* was more stable in floral tissue of *Anoectochilus roxburghi* (Lin *et al*., 2018). However, the screening of reference genes in *Helianthus tuberosus* showed that *25S RNA* was more stably expressed in petals and other tissues and could be used as a reference gene (Song *et al*., 2018). Other studies have found that *β-TUB* can be stably expressed in flower of *Bupleurum chinense*, while the *18S RNA* gene is more unstably expressed (Dong, 2008). The expression stability of the *TUB* reference gene is the best in flower of *Polygonatum sibiricum* and it is a reference gene (Wang *et al*., 2017).

By using different reference genes (*MDH*, *GADPH*, *18S RNA*, *ACT*, *UBQ*) of *A. vilmorinianum*, the expression levels of the *HMGCR* and *PMK* target genes showed that stably expressed reference genes can be used for standardization of target genes. However, in different tissues, the results of standardization of the expression level of target genes by selecting reference genes are different. In the same tissue, many stably expressed reference genes can be used as reference genes. Overall, these results indicate that the expression level of reference genes varies in different species and tissues, and usually the expression level varies greatly (Chapman *et al*., 2015). Therefore, with the change of experimental materials, it is very important to select appropriate reference genes to analyze the expression level of target genes.

Conclusion

As far as we know, this is the first systematic study on the identification of the reference genes of *Aconitum vilmorinianum* in root, stem, leaf and flower tissues by RT-qPCR analysis. The evaluation of six candidate reference genes showed that *MDH*, *GAPDH* and *18S RNA* could be used as reference genes in root; *MDH* and*18S RNA* could be used as reference genes in stem; *MDH*, *GAPDH* and *β-TUB* could be used as reference genes in leaf, and *GAPDH*, *18S RNA* and *ACT* could be used as reference genes in flower. In addition, the applicability of the reference genes selected in this study was verified by the analyzing the expression profiles of the target genes *HMGCR* and *PMK*. The results showed that the expression profiles of *HMGCR* and *PMK* genes were similar after standardization to stably expressed *MDH*, *GADPH*, 1*8S RNA* and *ACT* reference genes. However, when *UBQ* was used as reference gene, the expression profiles of *HMGCR* and *PMK* were different. These results will be helpful to obtain reliable standardized RT-qPCR data in the study of gene expression in *A. vilmorinianum*.

Acknowledgement

This work was supported in part by the National Naturel Science Foundation of China (No. 31560351; No. 31760349).

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(Received for publication 3 February 2021)